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Introduction

The development of methods for the preparation of α-lactalbumin from cow's milk whey (1–4) has made this crystalline, homogeneous, low-molecular-weight protein available for further investigation. In previous papers we have reported that α-lactalbumin contains about 7% tryptophan (1) and that the total sulfur of the protein is present in the form of 6.4% cystine and 0.95% methionine (2). Amino acid analyses of this protein by the chromatographic technique of Moore and Stein with Dowex 50 resin[2] (5) are described in the present paper.

Recent publications by Harfenist (6), Hirs (7), and Smith, Stockell and Kimmel (8, 9) have emphasized the importance in protein analysis of using various periods of hydrolysis in order to assure complete liberation of certain amino acids, such as valine and isoleucine, and to estimate more accurately the progressive decomposition of others, such as serine and threonine. In the analyses of α-lactalbumin to be described the protein was hydrolyzed for periods of 20, 70, and 140 hr. The longer periods were required for maximal liberation of valine and isoleucine, and a progressive decrease in tyrosine with increasing time was observed. We found serine and threonine to be completely stable in our experiments even when the protein was hydrolyzed for 140 hr.

Experimental

The α-lactalbumin was prepared by our first method (1). The crystalline protein was recrystallized several times and was electrophoretically homogeneous under the conditions previously described. It was then dialyzed and dried from the

[1] A laboratory of the Eastern Utilization Research Branch, Agricultural Research Service, U. S. Department of Agriculture.

[2] Mention of products in this paper does not imply endorsement or recommendation by the Department of Agriculture over similar products not mentioned.

frozen state. The protein was equilibrated with moisture and stored in a desiccator maintained at constant humidity. The weights of samples taken for analysis were corrected accordingly.

Samples of about 60 mg. protein were weighed out into small round-bottom flasks which could later be attached to the rotary concentration apparatus of Craig, Gregory, and Hausmann (11). A 200-fold quantity of glass-redistilled 6 *N* HCl was added, and hydrolysis was carried out for 20, 70, or 140 hr. under reflux at 120°. Little insoluble humin was formed under these conditions. Following hydrolysis, HCl was removed by concentration *in vacuo* at 37° in the Craig apparatus. The residue was dissolved in the appropriate Moore and Stein buffer (pH 3.4 for the 100-cm. column and 5.0 for the 15-cm. column runs) and diluted to volume. An aliquot containing about 5 mg. amino acids was then pipetted into the resin column.

Several samples of α-lactalbumin were oxidized with performic acid prior to hydrolysis to convert cystine to cysteic acid (12) and thus to improve the separation of the alanine peak in the chromatographic analysis.

The chromatographic separation of amino acids on long and short columns of Dowex 50 and analysis of the eluted fractions were carried out according to the method of Moore and Stein (5). Analysis of known amino acid mixtures gave excellent results. The separation of peaks was uniformly good except that there was some overlap of the alanine and cystine peaks. Using the ninhydrin color yields and the glutamic acid correction factor of Moore and Stein, satisfactory recoveries of all amino acids except lysine were obtained. In our hands the color yield of lysine, expressed as leucine equivalents, was 1.07 rather than 1.12. We have used the lower color yield in all our calculations since it enabled us to obtain quantitative results with known mixtures.

The separation of cystine became progressively worse with increased time of hydrolysis presumably due to racemization.[3] There was considerable progressive destruction of cystine and an apparent partial conversion to cysteic acid as judged by the size of the peak emerging from the column in the position of cysteic acid; therefore, quantitative determination of cystine was not possible. It was suspected that the broadness of the cystine peak might affect the alanine analyses. This was not the case as was demonstrated with hydrolyzates prepared after performic acid oxidation.

Amino nitrogen analyses were run on the intact protein by the Van Slyke method as modified by Doherty and Ogg (13). Amide nitrogen values were obtained by the method of Rees (10). Arginine analyses were run spectrophotometrically by the 8-oxyquinoline procedure of Sakaguchi (14). Tyrosine analyses were made by the spectrophotometric method of Udenfriend and Cooper (15). For the colorimetric determinations, 20-hr. periods of hydrolysis were used.

Results and Discussion

Analytical results from the 100-cm. column runs are listed in Table I. It will be noted that the threonine and serine values remained constant with increasing times of hydrolysis. In view of the data of Rees (10)

[3] Moore, S., personal communication.

TABLE I

Amino Acids Found in α-Lactalbumin Hydrolyzates

The amino acid values are given as grams amino acid yielded by 100 g. anhydrous ash-free protein. Hydrolyzate L-11 was prepared after oxidation with performic acid.

Amino acid	Time of hydrolysis					
	20 hr.		70 hr.		140 hr.	
	Hydrolyzates L-5 and L-6		Hydrolyzates L-7 and L-8		Hydrolyzates L-9 and L-11	
Aspartic acid	(17.69)[a]	18.45	18.89	18.80	18.45	
Threonine	5.43	5.39	5.54	5.63	5.51	
Serine	4.70	4.70	4.73	4.88	4.79	
Glutamic acid	12.61	12.73	13.00	13.01	12.91	
Proline	1.52	1.64	1.53	1.48	1.43	
Glycine	3.07	3.12	3.25	3.23	3.32	3.24
Alanine	2.01	2.13	2.13	2.10	2.34	2.15
Cystine	(4.37)	(5.60)	(3.58)	(4.33)	(3.05)	(6.66)[b]
Valine	(3.20)	(3.25)	4.60	4.52	4.89	4.62
Methionine	0.91	0.92	0.90	0.95	0.95	
Isoleucine	(5.41)	(5.53)	6.78	6.53	7.06	6.82
Leucine	11.09	11.44	11.67	11.54	11.93	11.43
Tyrosine	5.20	5.30	5.02	4.83	4.47	
Phenylalanine	4.36	4.27	4.47	4.62	4.64	

[a] Omitted because of operational difficulties.

[b] Estimated as cysteic acid but calculated as cystine, assuming a 90% conversion of cystine to cysteic acid during performic acid oxidation (12).

which led to the widespread acceptance of correction factors for the acid lability of serine and threonine, as well as the evidence from more recent investigations such as those of Smith *et al.* (8, 9), our results were entirely unexpected. We can offer no convincing explanation for the apparent stability of serine and threonine during the acid hydrolysis of α-lactalbumin. The yields of tyrosine decreased in linear fashion with time. Seventy hours of hydrolysis was needed for maximal yields of valine and isoleucine.

The cystine figures obtained from unoxidized protein have no quantitative significance for reasons previously mentioned. The value of 6.66% following performic acid oxidation and estimation as cysteic acid is in fair agreement with our colorimetric determination of 6.4% (2). The other results listed for performic-oxidized α-lactalbumin (hydroly-

zate L-11) agree well with analyses of the unoxidized protein. The alanine figure confirms the determinations for which some error was suspected because of spreading of the cystine peak. Not only were cystine and methionine oxidized by performic acid, but the peaks for tyrosine and phenylalanine disappeared almost completely in hydrolyzates of oxidized α-lactalbumin. Smith and Stockell found almost complete oxidation of tyrosine but less extensive destruction of phenylalanine following performic acid oxidation of carboxypeptidase (8), whereas Hirs reported that these amino acids were not affected in the oxidation of ribonuclease (7). It is apparent that the lability of tyrosine and phenylalanine during performic acid oxidation of proteins is determined by the conditions employed in the oxidation, by structural differences in proteins, or by a combination of these factors.

Results from the 15-cm. column and 20-hr. runs are presented in Table II. The low content of arginine in α-lactalbumin made accurate determinations difficult; therefore, independent colorimetric analyses were also run. These gave a value of 1.15% arginine as compared with the chromatographic figure of 1.05%. The higher estimate is believed to be more nearly correct on the basis of calculations of minimal molecular weight. The ammonia nitrogen content was 1.50%; amide nitrogen analyses gave 1.37%. A discrepancy of this order in these estimations can usually be accounted for in terms of serine and threonine destroyed during acid hydrolysis but cannot be so explained in the present work.

The amino acid composition of α-lactalbumin together with various calculations from the analytical data are summarized in Table III.

The tyrosine value of 5.37% was obtained by extrapolation of the data in Table I to zero time of hydrolysis from a line calculated by the method of least squares; analyses by the method of Udenfriend and Cooper (15) gave 5.2%. Figures in parentheses in Table I are omitted from the averages shown in Table III. The cystine, methionine, and

TABLE II

Basic Amino Acids of α-Lactalbumin as Determined in Three Different 20-hr. Hydrolyzates

Data are given as grams yielded by 100 g. anhydrous ash-free protein.

	Average, with average deviation
Histidine	2.85 ± 0.07
Lysine	11.47 ± 0.40
Ammonia	1.82 ± 0.02
Arginine	1.05 ± 0.07

TABLE III

Composition and Molecular Weight of α-Lactalbumin

Constituent	Grams/100 g. protein	N as % of total N, 15.86%[a]	Grams of amino acid residue/100 g. protein	Minimal mol. wt.	Calculated mol. wt.	Assumed no. of residues	Calcd. no. of residues for ave. mol. wt. of 15,554
Amino N	1.27			1,102	15,428	14	14.1
Amide N	1.37	8.64		1,022	15,330	15	15.2
Glycine	3.21	3.78	2.44	2,339	16,373	7	6.6
Alanine	2.14	2.12	1.71	4,163	16,652	4	3.7
Valine	4.66	3.51	3.94	2,514	15,084	6	6.2
Leucine	11.52	7.76	9.94	1,139	15,946	14	13.7
Isoleucine	6.80	4.58	5.87	1,930	15,440	8	8.1
Proline	1.52	1.17	1.28	7,574	15,148	2	2.1
Phenylalanine	4.47	2.39	3.98	3,696	14,784	4	4.2
Cystine	6.4[b]	4.71	5.44	3,755	15,020	4	4.1
Methionine	0.95[b]	0.56	0.84	15,706	15,706	1	1.0
Tryptophan	7.0[a]	6.06	6.38	2,917	14,585	5	5.3
Arginine	1.15[c]	2.33	1.03	15,148	15,148	1	1.0
Histidine	2.85	4.87	2.52	5,444	16,332	3	2.9
Lysine	11.47	13.86	10.06	1,275	15,300	12	12.2
Aspartic acid	18.65	12.37	16.13	714	15,708	22	21.8
Glutamic acid	12.85	7.71	11.28	1,145	16,030	14	13.6
Serine	4.76	4.00	3.95	2,208	15,456	7	7.0
Threonine	5.50	4.08	4.67	2,166	15,162	7	7.2
Tyrosine	5.37	2.62	4.84	3,374	16,870	5	4.6
Total	113.1[d]	97.12[e]	96.30		15,541[f] 15,554[g] 15,575[h]	126	125.3

[a] Values from previous analyses (1).

[b] Values from previous analyses (2).

[c] Average value (average deviation, ±0.08) from three determinations by method of Sakaguchi.

[d] Total includes amino acids and 1.82% ammonia.

[e] Total includes amide N.

[f] Average molecular weight for the seven amino acids present to the extent of 4 residues or less.

[g] Average molecular weight for the 14 amino acids present to the extent of 8 residues or less.

[h] Average molecular weight for all amino acids, amino N, and amide N (20 values).

tryptophan values used are those from our earlier analyses (1, 2) and the arginine figure is the afore-mentioned 1.15%. Calculations of the analytical data show that 97% of the total nitrogen can be accounted for, while the summation of amino acid residue weights is about 96%. From the amino acid residue weights, the specific volume of α-lactalbumin was computed (16). The calculated figure, 0.729 cc./g., is in good agreement with the experimentally determined value of 0.735 (1). We have no evidence on the distribution of amide groups relative to aspartic and glutamic acids in α-lactalbumin.

Calculations of the molecular weight of α-lactalbumin from the analytical data are also shown in Table III. The average molecular weight was found to be about 15,500 whether the calculation was based on the 7 amino acids present to the extent of 4 or less residues per mole, on the 14 amino acids present to the extent of 8 or less residues per mole, or on all 20 analytical determinations, including amino nitrogen and amide nitrogen. The calculations are based on the presence of one mole each of methionine and arginine per mole protein. The average molecular weight agrees quite well with the molecular weights of 15,100, calculated from sedimentation and diffusion measurements, and 16,500, calculated from light-scattering measurements (1).

The average molecular weight of 15,554 was used in calculating the number of residues of each component (Table III). The total number of amino acid residues is 125, or 129 when each cystine is considered as two residues.

TABLE IV

Distribution of Side Chain Groups in α-Lactalbumin

Total ionic, 18 + 23 = 41; total polar, 18 + 23 + 48 = 89; total nonpolar = 45.

Basic		Acidic		Nonionic polar		Nonpolar	
Arg	1	Asp	22	½Cys	8	Gly	7
His	3	Glu	14	Met	1	Ala	4
Lys	12	Free α-COOH	2	Try	5	Val	6
			38	Tyr	5	Leu	14
Free α-NH_2	2						
	18	Amide	−15	Ser	7	Iso	8
			23	Thr	7	Pro	2
				Amide	15	Phe	4
					48		45